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Non-functional expression of *Escherichia coli* signal peptidase I in *Bacillus subtilis*

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The *Escherichia coli lep* gene, encoding signal peptidase I (SPase I) was provided with *Bacillus subtilis* transcription/translation signals and expressed in this organism. When present on a low-copy-number plasmid, the amount of *E. coli* SPase I produced (per mg cell protein) in *B. subtilis* was half that produced in wild-type *E. coli* cells. The production of *E. coli* SPase I in *B. subtilis* was increased approximately fivefold by cloning the *lep* gene into a high-copy-number plasmid. The expression of *E. coli* SPase I in *B. subtilis* did not appear to increase the rate of processing of two hybrid secretory precursor proteins. Two observations may explain the failure of *E. coli* SPase I to stimulate processing of exported proteins in *B. subtilis*. First, the *E. coli* SPase I was apparently not exposed on the outside of the *B. subtilis* cytoplasmic membrane, indicating its incorrect insertion into the membrane. Second, *in vitro* processing studies, using cell-free extracts of *B. subtilis* producing *E. coli* SPase I, suggested that the enzyme was not active. A further outcome of this study was that conditions favouring processing of precursors by SPase I in cell-free extracts of *E. coli* did not favour processing by the corresponding enzyme in *B. subtilis* cell-free extracts. This suggests that significant differences exist between the two enzymes. The observation that antibodies directed against *E. coli* SPase I did not cross-react with *B. subtilis* membrane proteins supports this idea.

Introduction

The efficiency of bacterial protein export depends on properties of both the exported protein (for reviews, see Bankaitis *et al.*, 1987; Pollit & Inouye, 1987) and the cellular export machinery (for review, see Saier *et al.*, 1989).

Exported proteins are usually synthesized as precursors with an N-terminal extension, called the signal peptide (Watson, 1984). This signal peptide is involved in guiding the protein into the export pathway by interacting with the membrane and other components of the cellular export machinery (for reviews, see Pollit & Inouye, 1987; Randall & Hardy, 1989; Saier *et al.*, 1989). Moreover, the signal peptide retards the folding of the precursor, which appears to be essential for export-competence (Laminet & Plückthun, 1989; Liu *et al.*, 1988; Park *et al.*, 1988; for review, see Saier *et al.*, 1989). Cytoplasmic components, denoted as chaperones, are required for maintaining the export-competence of precursor proteins, either by stabilizing an unfolded conformation (see Saier *et al.*, 1989), or by preventing their aggregation (Lecker *et al.*, 1990; Mitraki & King, 1989). So far, three different chaperones have been described in *E. coli*: (1) SecB, (2) GroEL and (3) GroES (see Kusukawa *et al.*, 1989; Lecker *et al.*, 1989). Other cellular components required for protein translocation across the cytoplasmic membrane in *E. coli* are SecA, SecD, SecE and SecY (see Saier *et al.*, 1989). The final step in protein translocation is the release of the mature part of the protein from the membrane, which requires the proteolytic removal of the signal peptide. This processing step is catalysed by signal peptidases (SPases; for review, see Ray *et al.*, 1986). Two *E. coli* SPases have been cloned and sequenced: SPase I (synonymous for leader peptidase) is encoded by the *lep* gene (Date & Wickner, 1981; Wolfe *et al.*, 1983*a*) and SPase II (synonymous for prolipoprotein signal peptidase) is encoded by the *lsp* gene (Innis *et al.*, 1984; Yu *et al.*, 1984). SPase I is responsible for the processing of the precursor of bacteriophage M13 coat protein and the majority of exported pre-proteins (Dalbey & Wickner, 1985; Wolfe *et al.*, 1982). SPase II exclusively processes glyceride-modified lipoproteins (Tokunaga *et al.*, 1982; Yamada *et al.*, 1984).

Both SPase I and II are essential for viability, as demonstrated in mutant strains of *E. coli* producing low levels of either SPase I or II under certain conditions

Abbreviations: SPase, signal peptidase.

(Dalbey & Wickner, 1985; van Dijl *et al.*, 1988; Yamagata *et al.*, 1982). SPase limitation resulted in the accumulation of precursors of exported proteins.

Recently, we demonstrated that the availability of SPase I in *E. coli* could also be limiting under standard conditions (van Dijl *et al.*, 1991*a*): certain (hybrid) precursors of TEM-β-lactamase fused to signal peptides which had been randomly selected from the *B. subtilis* chromosome were inefficiently processed (Smith *et al.*, 1987, 1988, 1989; H. Smith and others, unpublished; van Dijl *et al.*, 1991*b*). The processing and export efficiencies of several of these precursors could be considerably improved by SPase I overproduction (van Dijl *et al.*, 1991*a*). In contrast, the processing rates of wild-type pre-β-lactamase and hybrid precursors, already high under standard conditions, were not detectably altered by SPase I overproduction.

In *B. subtilis* the processing of certain hybrid precursors containing randomly selected signal peptides was not efficient either (H. Smith and others; unpublished; van Dijl *et al.*, 1991*b*). We reasoned that the availability of SPase I might also be a limiting factor in the processing of these precursors in this organism. However, since *lep* genes from bacilli have not been cloned so far, it was not possible to address this question directly in a homologous system. Since *B. subtilis*-exported proteins are usually efficiently processed in *E. coli*, we reasoned that the *E. coli* SPase I might function in *B. subtilis*. This consideration prompted us to study the effects of *E. coli* SPase I production on the processing of export proteins in *B. subtilis*. The results show that, although the *E. coli* SPase I was produced by *B. subtilis*, *in vivo* processing efficiencies were not increased.

Methods

Bacteria and plasmids. The bacterial strains and plasmids used are listed in Table 1.

Media and plates. TY medium contained (g l^{-1}): Bacto tryptone, 10; Bacto yeast extract, 5; and NaCl, 10. S7 medium used in the pulse-chase labelling of *B. subtilis* DB114(pSB-A2d) was made according to Vasantha & Freese (1980) with the modification that MOPS was replaced by 20 mM-potassium phosphate (S7 medium-1). Since the synthesis of *B. licheniformis* α-amylase is subject to glucose repression in *B. subtilis*, the synthesis of this protein was achieved by growth of *B. subtilis* 8G5(*amy*)(pSA13) in S7 medium-2, in which glucose was replaced by 2% (w/v) starch (Merck) (H. Smith and others, unpublished). S7 media-3 and -4 were methionine-free variants of S7 media-1 and -2, respectively. In S7 medium-4 the amount of starch was reduced to 0·1% (w/v). If required, the media were supplemented with chloramphenicol (2 μg ml^{-1}), erythromycin (2 μg ml^{-1}) and kanamycin (10 μg ml^{-1}).

DNA techniques. Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of competent *E. coli* cells were carried out as described in Maniatis *et al.* (1982). Transformation of competent *B. subtilis* cells was performed as described by Bron & Venema (1972). Enzymes (Boehringer) were used as indicated by the supplier.

Western blot analysis. The expression of SPase I was assayed by Western blotting (Towbin *et al.*, 1979) on nitrocellulose membranes (BA 85, Schleicher and Schuell). SPase I production was monitored with specific antibodies (obtained from Dr R. Zimmermann, Universität München, FRG) and subsequent visualization of the bound antibodies was achieved with alkaline phosphatase anti-rabbit IgG conjugates (Protoblot, Western Blot AP system, Promega). Reference SPase I was purified from an overproducing strain as described by Wolfe *et al.* (1983*b*).

Protein assay. Protein was quantified by the method of Bradford (1976).

Pulse-chase protein labelling. Exponential phase cells in S7 medium-1 or -2 were washed once with methionine-free S7 medium-3 or -4, respectively, and incubated for 45 min at 37 °C in these media. Labelling with [^{35}S]methionine (1330 Ci $mmol^{-1}$, 49·2 TBq $mmol^{-1}$; Amersham) for the times indicated, chasing with excess (2·5 mg ml^{-1}) non-radioactive methionine and sampling, followed by the immediate precipitation of proteins with trichloroacetic acid (TCA) at 0 °C, were performed as described previously (van Dijl *et al.*, 1988). Precipitates were resuspended in 100 μl 10 mM-Tris/HCl (pH 8·0), 25 mM-$MgCl_2$, 200 mM-NaCl and 5 mg lysozyme ml^{-1} (Boehringer). After 10 min at 37 °C, lysis was completed by the addition of 10 μl 10% (w/v) SDS and heating for 10 min at 70 °C.

Immunoprecipitation, SDS-PAGE and fluorography. Immunoprecipitation was carried out as described by Edens *et al.* (1982) with specific antisera. SDS-PAGE was performed according to Laemmli (1970). ^{14}C-Methylated molecular mass reference markers were obtained from Amersham. Fluorography was performed as described by Skinner & Griswold (1983). Relative amounts of radioactivity (pulse-chase experiments), or of alkaline phosphatase staining (Western blot analysis) were estimated by densitometer scanning with an LKB Ultroscan XL enhanced laser densitometer.

Isolation of B. subtilis membranes. Exponential phase cells in TY medium were concentrated 40-fold in 100 mM-potassium phosphate buffer (pH 6·6) and 10 mM-EDTA. The cell suspension was incubated for 10 min at 37 °C in the presence of lysozyme (1 mg ml^{-1}). Lysis was completed using a French press at 6000 p.s.i. (41·3 MPa) (0 °C). Unlysed cells were removed by low-speed centrifugation (SW 50.1 rotor, 1500 r.p.m., 15 min, 4 °C). Membranes were subsequently separated from cytoplasmic contents by centrifugation (150000 ***g***, 1 h, 4 °C) and resuspended in one-third vol. of the same buffer, with or without 1% (v/v) Triton X-100.

Spheroplasting of E. coli. Exponential phase cells of *E. coli* in TY medium were concentrated 10-fold in spheroplast-buffer [30 mM-Tris/HCl buffer (pH 8·0), 20% (w/v) sucrose, 10 mM-EDTA, 0·5 mg lysozyme ml^{-1}) and incubated for 30 min at 37 °C. Spheroplasts were directly used for further experiments.

Protoplasting of B. subtilis. Exponential phase *B. subtilis* DB114-15 cells in TY medium were concentrated 10-fold in spheroplast-buffer and incubated for 30 min at 37 °C. Protoplasts were directly used for further experiments.

In vitro transcription/translation. ^{35}S-Labelled precursors of the bacteriophage M13 coat protein and the outer-membrane protein PhoE were synthesized *in vitro* as described by de Vrije *et al.* (1987).

In vitro processing. Exponential phase cultures of *E. coli* or *B. subtilis* in TY medium were concentrated 40-fold in 50 mM-triethanolamine/HCl (pH 8·0), 1 mM-EDTA, 20% (w/v) sucrose and 1 mg lysozyme ml^{-1}, and the suspension was incubated for 20 min at room temperature. Lysis was completed using a French press at 6000 p.s.i. (41·3 MPa) in the same buffer containing 1 mM-dithiothreitol. Triton X-100 was added to the extracts to a final concentration of 1% (v/v). Processing reactions were carried out by incubating 4 μl of a five-times

Table 1. *Bacterial strains and plasmids*

Plasmid or strain	Properties and genotype	Source or reference
Plasmid		
pTD101	pBR322 carrying the *Escherichia coli lep* operon; 8·9 kb, Ap^R	Date & Wickner (1981)
pUC9/pUC18	2·8 kb, Ap^R	Vieira & Messing (1982)
pHP13	pTA1060–pUC9 derivative; 4·9 kb, Cm^R Em^R	Haima *et al.* (1987)
pHP13-2	Deletion derivative of pHP13; 4·7 kb, Cm^R Em^R	Haima *et al.* (1990*a*)
pPL608	5·1 kb, Cm^R Km^R; bacteriophage SPO2 promoter	Williams *et al.* (1981)
pHPS4	pHP13-2 derivative carrying a *cat-86*::*lacZα* gene fusion; bacteriophage SPO2 promoter; 5·4 kb, Cm^R Em^R; five copies per chromosome equivalent	Laboratory collection (P. Haima)
pHPS41	pHPS4 derivative; Em^R	This paper
pHPL0	pUC18 carrying an 811 bp *Nla*III fragment encoding the 5′-end of the *lep* gene; 3′ end of *lep* gene is truncated; 6·1 kb, Cm^R Em^R	This paper
pHPL1	pHPS4 carrying a *cat-86*::*lep* gene fusion; 7·9 kb, Cm^R Em^R	This paper
pHPL2	pHPL1 derivative; Em^S	This paper
pGD40	pSC105-derived cloning vector; 8·4 kb, Km^R Tc^R *cI857*$^+$	van Dijl *et al.* (1990)
pGDL20	pGD40 carrying a *cat-86*::*lep* gene fusion; 12·3 kb, Km^R Tc^R *cI857*$^+$	This paper
pTZ12	2·5 kb, Cm^R; 150–200 copies per chromosome equivalent	Aoki *et al.*, (1987)
pGDV1	pTZ12-derived cloning vector; 2·6 kb, Cm^R	Bron (1990)
pGDL24	pGDV1 containing the *cat-86*::*lep* gene fusion of pHPL1; 6·4 kb, Cm^R	This paper
pGDL2	pSC101-derived plasmid carrying the *lep* gene under the control of the *tac* promoter; 9·8 kb, Km^R	van Dijl *et al.* (1991*a*)
pGPA14	α-Amylase based signal sequence selection vector carrying the pTA1060 replication functions	Smith *et al.* (1987)
pSPA13	pGPA14 carrying signal sequence A13; amylase$^+$ Em^R	Smith *et al.* (1988)
pSA13	pSPA13 with the pTA1060 replication functions replaced by those from the lactococcal plasmid pWVO1; compatible with pHPS41- and pGDV1-derived plasmids	Laboratory collection
pGPB14	β-Lactamase based signal sequence selection vector carrying the pTA1060 replication functions	Smith *et al.* (1987)
pSPB-A2d	pGPB14 carrying signal sequence A2d; Em^R	H. Smith and others (unpublished)
pSB-A2d	pSPB-A2d with the pTA1060 replication functions replaced by those of pWVO1; compatible with pHPS41- and pGDV1-derived plasmids	Laboratory collection
Strains		
Bacillus subtilis		
8G5	*trpC2 tyr his nic ura rib met ade*	Bron & Venema (1972)
8G5(*amy*)	α-Amylase-negative derivative of 8G5	Laboratory collection
6GM15	*trpC2 tyr his ura rib met lacZ*ΔM15 Km^R	Haima *et al.* (1990*b*)
DB104	*his nprR2 nprE18 aprA3*	Kawamura & Doi (1984)
DB114	*met nprR2 nprE18 aprA3*	Laboratory collection
DB114-15	Derivative of DB114; *lacZ*ΔM15 Km^R	This paper
Escherichia coli		
C600	*thr leu thi lacY tonA phx supE vtr*	Phabagen collection, State University, Utrecht, The Netherlands
JM83	*ara* Δ*lac–proAB thi strA* φ80 *lacZ*ΔM15	Vieira & Messing (1982)
N4156::pGD28	*polA end thy gyrA*: *lep* gene under transcriptional control of λp_L: Ap^R	van Dijl *et al.* (1988)
N4156::pGD28 (*cI857*)	Unable to grow at 28 °C: Ap^R	van Dijl *et al.* (1988)

Ap, ampicillin; Cm, Chloramphenicol; Em, erythromycin; Km, kanamycin; Tc, tetracycline.

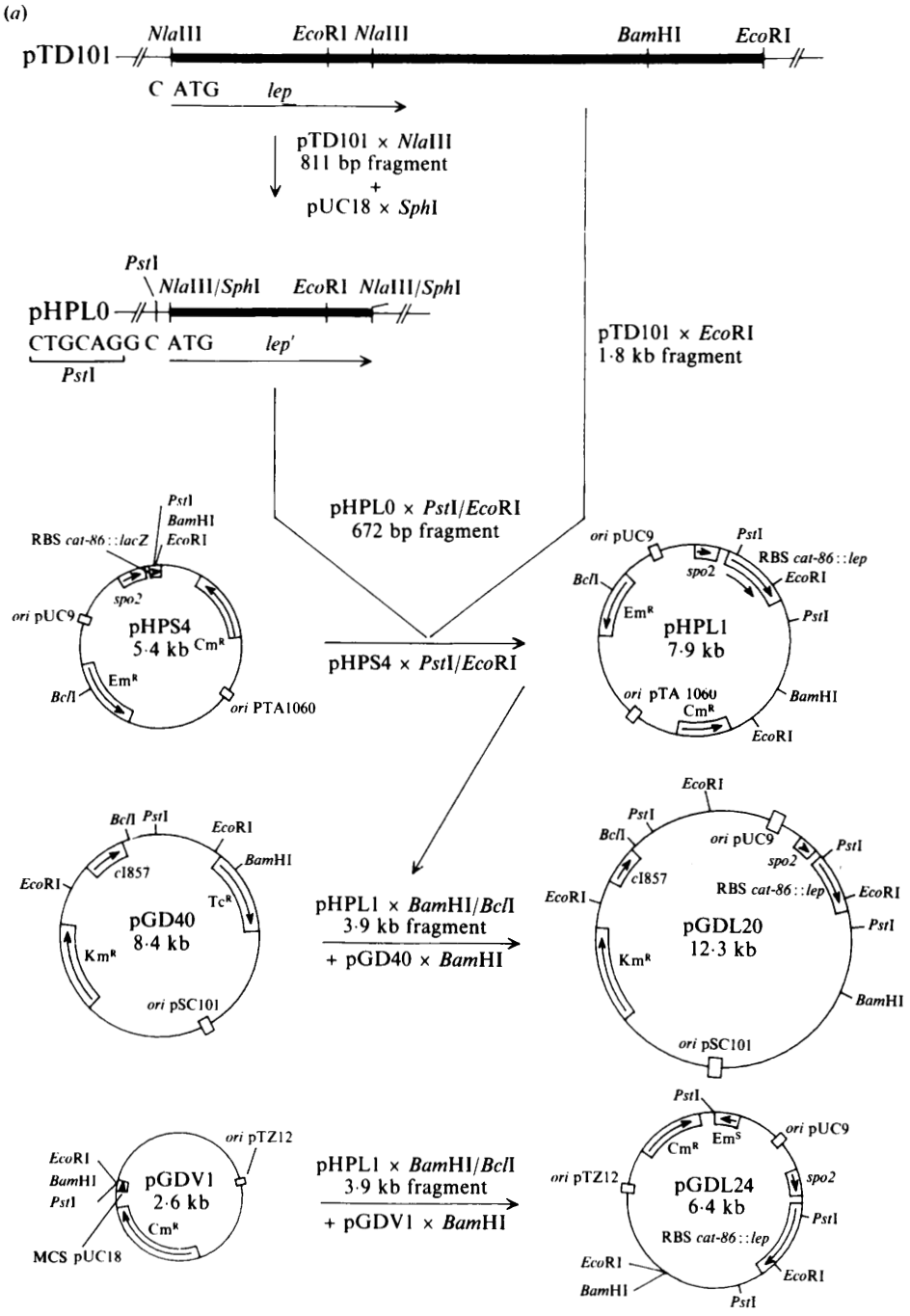
diluted *in vitro* transcription/translation mixture in 50 mM-triethanolamine/HCl; (pH 8·0), 1 mM-EDTA, 1% (v/v) Triton X-100, 1 mM-PMSF with various amounts of extract, or with purified SPase I at 37 °C for 60 min (total reaction volume 14 μl).

Results

Fusion of the E. coli lep gene to B. subtilis gene expression sequences

To express the *E. coli lep* gene in *B. subtilis*, it was placed under the control of efficient transcription/translation signals for this organism. This was accomplished by using the bacteriophage SPO2 promoter and the Shine–Dalgarno sequence of the *B. pumilus cat-86* gene (Williams *et al.*, 1981), which are present on the low-copy-number plasmid pHPS4 (five copies per chromosome equivalent; Fig. 1*a*). The resulting plasmid, pHPL1, contained an in-frame fusion between the first two codons of the *cat-86* gene, five codons derived from the multiple-cloning-site of pUC9, and the complete *lep gene* (*cat-86*::*lep*, Fig. 1*b*).

To examine whether the hybrid SPase I protein was active, a complementation analysis was performed in *E. coli* N4156::pGD28. In this strain the chromosome-



(*b*)

		PstI	NlaIII
SPO2 promoter	Shine–Dalgarno sequence *cat-86*	TTG TTT AGC TTG GCT GCA GGC ATG Met Phe Ser Leu Ala Ala Gly Met	

located *lep* gene is transcribed from the repressible phage λ p_L promoter (van Dijl *et al.*, 1988). SPase I synthesis in this strain can be controlled by the λcI857 temperature-sensitive repressor, provided in *trans* via the signal peptidase probe vector pGD40. Under conditions of repression of SPase I synthesis (28 °C), strain N4156::pGD28 is unable to grow. Cloning of *lep* genes into pGD40 results in the restoration of growth at 28 °C,

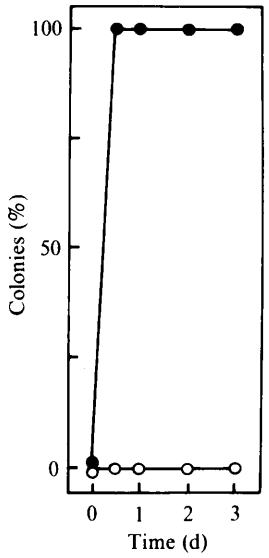


Fig. 2. Growth of *E. coli* N4156::pGD28(pGD40, pGDL20) at 28 °C. The viability of 50 transformants of each class obtained at 28 °C was tested by transfer to fresh plates and incubation at 28 °C. The percentage of transfers that developed into colonies was determined as a function of time of incubation. ○, pGD40, ●, pGDL20.

provided that the product of the cloned *lep* gene is functional (van Dijl *et al.*, 1990). In the test system described here, the 3·9 kb *BclI–Bam*HI fragment from plasmid pHPL1, containing the hybrid *lep* gene, was ligated into the unique *Bam*HI site of pGD40. This resulted in pGDL20 (Fig. 1*a*). The viability of *E. coli* N4156::pGD28 after transformation with pGD40 and pGDL20 was tested at 28 and 42 °C. As expected, at 42 °C (chromosomal *lep* gene expressed) transformants carrying pGD40 or pGDL20 showed normal growth properties (data not shown). In contrast, at 28 °C (chromosomal *lep* gene repressed) transformants carrying pGD40 had lost their colony-forming capacity when transferred to fresh plates, whereas transformants carrying pGDL20 did form colonies (Fig. 2). This test has been used successfully before (van Dijl *et al.*, 1990) to demonstrate the presence of a functional *lep* gene product. Similar results were obtained when transformants were transferred to liquid medium:

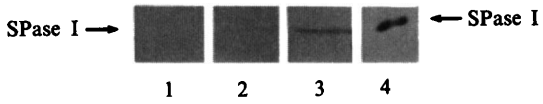


Fig. 3. Production of *E. coli* SPase I in *B. subtilis*. *B. subtilis* DB114-15, transformed with pHPS4, pHPL1 or pGDL24 was grown in TY medium. Exponential phase cells were lysed in buffer containing 0·1 M-potassium phosphate (pH 7·2) and 1 mg lysozyme ml^{-1}. Similar amounts (approximately 0·03 mg total protein) were subjected to SDS-PAGE and Western blotting. SPase I was detected with specific antisera. Lanes: 1, *B. subtilis* DB114-15(pHPS4); 2, *B. subtilis* DB114-15(pHPL1); 3, *B. subtilis* DB114-15(pGDL24); 4, reference SPase I.

N4156::pGD28(pGD40) did not grow at 28 °C, whereas normal growth of N4156::pGD28(pGDL20) was observed (data not shown). These results indicate that the SPase I encoded by pHPL1/pGDL20 complemented the defect in strain N4156::pGD28 and, therefore, that the hybrid SPase I was active in *E. coli*.

Expression of E. coli SPase I in B. subtilis

The production of *E. coli* SPase I in *B. subtilis* DB114(pHPL1) was monitored by Western blotting (Fig. 3, lane 2), which showed that the production of the enzyme (per mg total protein) in exponential phase cells was approximately 50% of the enzyme produced in wild-type (plasmid-free) cells of *E. coli* C600 (data not shown). In an attempt to raise the production of *E. coli* SPase I in *B. subtilis*, the 3·9 kb *BclI–Bam*HI fragment carrying the hybrid *lep* gene was ligated into the unique *Bam*HI site of the high-copy-number plasmid pGDV1 (150–200 copies per chromosome equivalent). This resulted in pGDL24 (Fig. 1). As compared to *B. subtilis* cells containing pHPL1, cells containing pGDL24 produced approximately five-times more SPase I (Fig. 3, lane 3).

Effects of E. coli SPase I production on the in vivo processing of hybrid precursor proteins in B. subtilis

The effects of the production of *E. coli* SPase I in *B. subtilis* on the processing kinetics of two hybrid precursors, pre(A13)-α-amylase and pre(A2d)-β-lacta-

Fig. 1. (*on facing page*) (*a*) Scheme of the construction of pHPL1, pGDL20 and pGDL24. Only restriction sites relevant for the construction and properties of the plasmids are shown. To provide the *lep* gene with the Shine–Dalgarno sequence of the *B. pumilus cat-86* gene, resulting in a *cat-86*::*lep* gene fusion, several construction steps were required. First, an 811 bp pTD101-derived *Nla*III fragment, containing the 3′-truncated *E. coli lep* gene (*lep′*), was ligated into the unique *Sph*I site of pUC18. This resulted in plasmid pHPL0. Plasmid pHPS4 was obtained by cloning a 700 bp *Dra*I fragment, derived from plasmid pPL608 (Williams *et al.*, 1981), in plasmid pHP13-2, which resulted in an in-frame fusion between the first two codons of *cat-86* and α-*lacZ* (P. Haima, unpublished results). From plasmid pHPL0 the 5′ end of the *lep* gene, present on a 672 bp *Pst*I–*Eco*RI fragment, was isolated. This fragment and a 1·8 kb pTD101-derived *Eco*RI fragment, containing the 3′ end of the *lep* gene were ligated into plasmid pHPS4, cleaved with *Pst*I and *Eco*RI, which resulted in pHPL1. pHPL1 contained an in-frame fusion of the 5′ end of the *lep* gene to the first two codons of the *cat-86* gene (the sequence of the fusion site is shown in *b*). pGDL20 and pGDL24 were constructed by ligating the 3·9 kb *Bam*HI–*Bcl*I fragment of pHPL1 containing the *cat-86*::*lep* gene fusion into the unique *Bam*HI sites of pGD40 and pGDV1, respectively. MCS, multiple cloning site; RBS, ribosome binding site. (*b*) Nucleotide sequence of the *cat-86*::*lep* gene fusion and the deduced amino acid sequence.

mase, were studied by pulse-chase labelling experiments. Both hybrid precursors contain signal peptides which had been randomly selected previously from the *B. subtilis* chromosome (Smith *et al.*, 1987, 1988, 1989). Under standard conditions pre(A13)-α-amylase was processed relatively slowly in *B. subtilis*: the time required to process 50% of the precursor (t_{50}) was approximately 5 min (H. Smith and others, unpublished; van Dijl *et al.*, 1991*b*). In contrast, the same precursor was processed very rapidly in *E. coli* (no precursor could be detected; H. Smith and others, unpublished; van Dijl *et al.*, 1991*b*). Assuming that the different processing rates in these two organisms reflect differences in the interaction of pre(A13)-α-amylase with the respective SPases, rather than differences in translocation efficiencies, we reasoned that pre(A13)-α-amylase might be a suitable precursor to detect possible activity of *E. coli* SPase I in *B. subtilis*. Pre(A2d)-β-lactamase was also processed relatively slowly in *B. subtilis* ($t_{50} = 2$ min; H. Smith and others, unpublished; van Dijl *et al.*, 1991*b*). In contrast to pre(A13)-α-amylase, this precursor was processed extremely slowly in *E. coli* under standard conditions of SPase I production (H. Smith and others, unpublished; van Dijl *et al.*, 1991*a*). Interestingly, overproduction of SPase I in *E. coli* increased the rate of pre(A2d)-β-lactamase processing drastically ($t_{50} < 1$ min; van Dijl *et al.*, 1991*a*). Based on this result, we reasoned that in addition to pre(A13)-α-amylase, pre(A2d)-β-lactamase might be a suitable substrate for detecting the activity of *E. coli* SPase I in *B. subtilis*.

Strains of *B. subtilis* producing either pre(A13)-α-amylase or pre(A2d)-β-lactamase were transformed with pHPS41 or pGDV1 (no production of *E. coli* SPase I), pHPL2 (low level of *E. coli* SPase I production) or pGDL24 (high level of *E. coli* SPase I production). Plasmids pHPS41 and pHPL2 are similar to pHPS4 and pHPL1, respectively, but lack a functional erythromycin-resistance gene. This enabled the co-selection of transformants containing both pHPS41 (or pHPL2) and the erythromycin-marked plasmid encoding the precursor of interest (pSA13 or pSB-A2d). The results of the pulse-chase labelling experiments showed that the processing rates of both precursors were not increased in the presence of pHPL2 (Fig. 4) or pGDL24 (data not shown), suggesting that the activity of the *E. coli* SPase I was too low to be detected in this assay.

Localization of E. coli SPase I in B. subtilis

In *E. coli*, SPase I spans the cytoplasmic membrane twice and most of the protein (the C-terminal part) is exposed to the outer surface of the membrane (Dalbey *et al.*, 1987; Moore & Miura, 1987). Treatment of spheroplasted *E. coli* cells with trypsin caused the digestion of the exposed

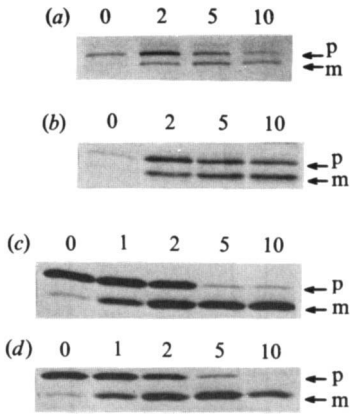


Fig. 4. Processing of pre(A13)-α-amylase (*a*, *b*) and pre(A2d)-β-lactamase (*c*, *d*). The kinetics of processing were analysed by pulse-chase labelling of the cells for 1 min at 37 °C, followed by immunoprecipitation of the protein studied, SDS-PAGE and fluorography. Samples were taken after the chase ($t = 0$) at the times indicated (min). (*a*) *B. subtilis* 8G5(*amy*) (pSA13, pHPS41); (*b*) *B. subtilis* 8G5(*amy*)(pSA13, pHPL2); (*c*) *B. subtilis* DB114(pSB-A2d, pHPS41); (*d*) *B. subtilis* DB114(pSB-A2d, pHPL2). p, Precursor; m, mature.

SPase I moiety. An N-terminal fragment of SPase I, designated TRF II, remained protected against degradation (Wolfe *et al.*, 1983*a*; Moore & Miura, 1987). Since incorrect membrane assembly is a possible explanation for the observed lack of *E. coli* SPase I activity in *B. subtilis*, we studied the localization properties of this enzyme in *B. subtilis* membranes in two different ways. First, membrane and cytoplasmic fractions of *B. subtilis* DB114 containing either pGDV1 or pGDL24 were isolated. No *E. coli* SPase I was detectable in the cytoplasmic fractions (data not shown). The enzyme was detectable only in the membrane fraction of cells containing pGDL24. Like in *E. coli*, the SPase I was solubilizible in 1% (v/v) Triton X-100 (Fig. 5, lane 5). Fig. 5 also shows that antibodies directed against the *E. coli* SPase I cross-react weakly with a membrane protein of *B. subtilis* of a higher molecular mass than that of *E. coli* SPase I (Fig. 5, lanes 2–5). Since this protein was not detected with a different batch of antibodies directed against SPase I (data not shown) we concluded that this cross-reactivity was not SPase-I-specific.

The question as to how the *E. coli* SPase I was present in the *B. subtilis* membrane was addressed by trypsin treatment of protoplasts of *B. subtilis* DB114-15 cells containing either pHPL1 (data not shown) or pGDL24 (Fig. 6*a*). Cells containing pGDV1 were used as controls. The LacZΔM15 protein produced by DB114-15 served as a cytoplasmic reference protein (Fig. 6*a*2). The results showed that, unlike the situation described for *E. coli*, digestion of SPase I was not detectable upon treatment of *B. subtilis* protoplasts with trypsin (Fig. 6*a*1, lane 3).

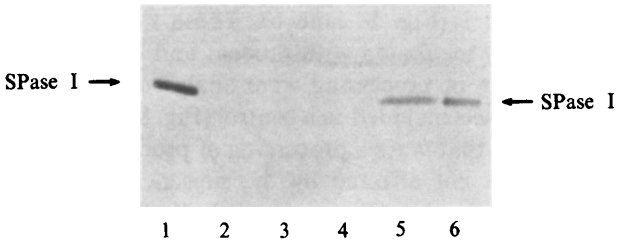


Fig. 5. Membrane association and solubilization of *E. coli* SPase I in *B. subtilis* DB114. Membranes of *B. subtilis* DB114(pGDV1) and *B. subtilis* DB114(pGDL24) were prepared as described in Methods. Membrane proteins were solubilized by the addition of 1% (v/v) Triton X-100 and incubation for 15 min (0 °C). Solubilized membrane proteins were separated from non-solubilized membrane proteins by centrifugation (1 h, 4 °C, 150000 *g*). The non-solubilized membrane proteins were resuspended in an equal volume of the same buffer. Equal amounts of both fractions were used for SDS-PAGE and the presence of SPase I was monitored by Western blotting. Lanes: 1 and 6, reference SPase I; 2, non-solubilized proteins of *B. subtilis* DB114(pGDV1); 3, solubilized proteins of *B. subtilis* DB114(pGDV1); 4, non-solubilized proteins of *B. subtilis* DB114(pGDL24); 5, solubilized proteins of *B. subtilis* DB114(pGDL24).

Treatment of the protoplasts with trypsin did not cause the disruption of the protoplast, since no digestion of the LacZΔM15 protein was detectable (Fig. 6*a*2, lane 3). Only when the protoplasts were disrupted with 1% (v/v) Triton X-100 and then treated with trypsin were the SPase I (Fig. 6*a*1, lane 4) and the LacZΔM15 protein (Fig. 6*a*2, lane 4) degraded to any extent. These results suggest that the *E. coli* SPase I was not incorporated into the *B. subtilis* membrane in such a way that potential sites for cleavage by trypsin were exposed on the outside of the protoplasts, suggesting that SPase I was incorrectly inserted in the membrane. In a control experiment, intact and disrupted spheroplasts of *E. coli* C600(pGDL2), which overproduces SPase I (van Dijl *et al.*, 1991*a*), were also treated with trypsin (Fig. 6*b*). As expected, in this case treatment of intact spheroplasts resulted in the partial degradation of SPase I to TRF II (Fig. 6*b*, lane 2). In the presence of 1% (v/v) Triton X-100, SPase I was completely degraded (Fig. 6*b*, lane 3).

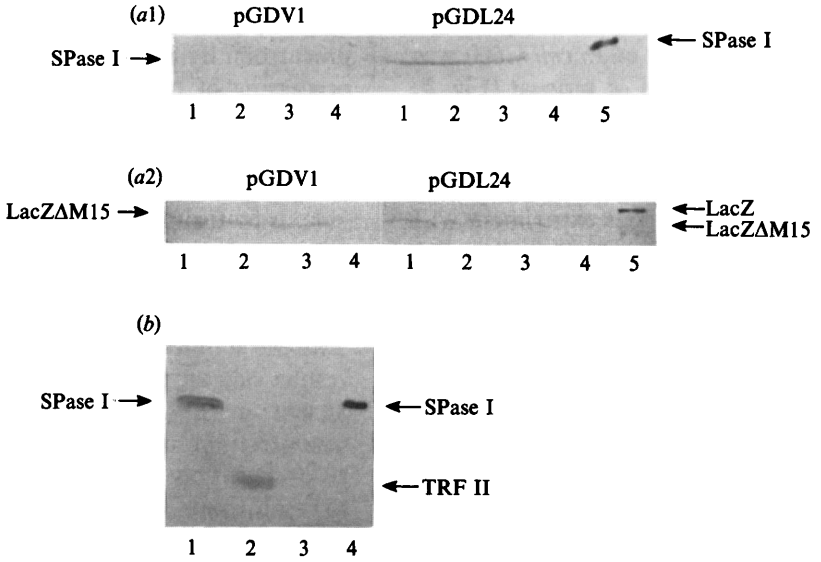


Fig. 6. Localization of *E. coli* SPase I in *B. subtilis* (*a*) and in *E. coli* (*b*). (*a*) Cells of *B. subtilis* DB114-15 were protoplasted as described in Methods. In separate experiments, intact and disrupted protoplasts of cells containing either pGDV1 or pGDL24, were incubated with trypsin (10 μg ml^{-1}) for 30 min at room temperature. Protoplasts were disrupted in 1% (v/v) Triton X-100 prior to treatment with trypsin. Prior to analysis by SDS-PAGE and Western blotting, samples were treated with trypsin-inhibitor (30 μg ml^{-1}). The presence of SPase I (*a*1) or the LacZΔM15 protein (*a*2) in samples (approximately 0·03 mg of total protein) was monitored with specific antibodies. Lanes: 1, unprotoplasted cells; 2, intact untreated protoplasts; 3, protoplasts incubated with trypsin; 4, protoplasts incubated with trypsin in the presence of 1% (v/v) Triton X-100; 5 (*a*1), reference SPase I; 5 (*a*2), reference LacZ protein. (*b*) Cells of *E. coli* containing plasmid pGDL2 were spheroplasted as described in Methods. Intact and disrupted spheroplasts were incubated with trypsin (10 μg ml^{-1}) for 30 min at room temperature. Spheroplasts were disrupted in 1% (v/v) Triton X-100 prior to treatment with trypsin. Prior to analysis by SDS-PAGE and Western blotting, samples were treated with trypsin-inhibitor (30 μg ml^{-1}). The presence of SPase I and TRF II in samples (approximately 0·02 mg total protein) was monitored with specific antibodies. Lanes: 1, intact untreated spheroplasts; 2, spheroplasts incubated with trypsin; 3, spheroplasts incubated with trypsin in the presence of 1% (v/v) Triton X-100; 4, reference SPase I.

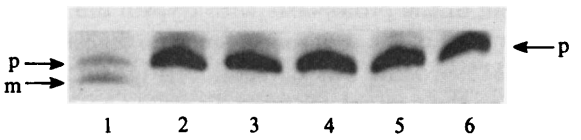


Fig. 7. *In vitro* processing of bacteriophage M13 procoat. *In vitro* synthesized M13 procoat was incubated with cell-free extracts from *E. coli* (0·4 mg protein ml^{-1}) and various strains of *B. subtilis* (4 mg protein ml^{-1}). Lanes: 1, *E. coli* C600; 2, no extract added; 3, *B. subtilis* DB114(pHPS4); 4, *B. subtilis* DB114(pHPL1); 5, *B. subtilis* DB114(pGDV1); 6, *B. subtilis* DB114(pGDL24). p, Precursor; m, mature.

In vitro activity of the E. coli SPase I produced in B. subtilis

To examine whether the *E. coli* SPase I produced in *B. subtilis* was active, *in vitro* synthesized bacteriophage M13 coat protein precursor (procoat) was incubated with cell-free extracts of *B. subtilis* containing pHPL1 (Fig. 7, lane 4) or pGDL24 (Fig. 7, lane 6). As a control, procoat was also incubated with cell-free extracts of *B. subtilis* containing pHPS4 (Fig. 7, lane 3) or pGDV1 (Fig. 7, lane 5) and with a cell-free extract of *E. coli* C600 producing standard amounts of SPase I (Fig. 7, lane 1). The results show that only the cell-free extract of *E. coli* C600 was able to process significant amounts of procoat (Fig. 7, lane 1). None of the *B. subtilis* extracts were able to process M13 procoat, even under conditions in which 10-fold higher amounts of extract (4 mg protein ml^{-1}) were added in comparison to the analogous experiment with *E. coli* extracts (0·4 mg protein ml^{-1}). This suggests that the enzyme synthesized in *B. subtilis* was not active.

It was apparent from Fig. 7 that the endogenous *B. subtilis* SPase I was likewise unable to process M13 procoat under the conditions used (lanes 3–6). This might be due to the presence in *B. subtilis* cell-free extracts of unknown components interfering with productive processing of procoat by both the *E. coli* and the *B. subtilis* SPase I. To test this, cell-free extracts from *B. subtilis* were mixed with extracts from *E. coli* C600 (producing standard levels of SPase I; Fig. 8, lane 5) and with cell-free extracts from *E. coli* C600(pTD101), which overproduces SPase I (Fig. 8, lane 6). These mixtures were subsequently incubated with procoat and the effects on the efficiency of processing were analysed. Purified *E. coli* SPase I was included as a control (Fig. 8, lane 7). The results show that *in vitro* processing of procoat by *E. coli* SPase I was not affected by the presence of cell-free extract of *B. subtilis* (Fig. 8, lanes 5–7). This indicates that the *B. subtilis* extracts did not interfere with *E. coli* SPase I activity and, therefore, it is concluded that the *E. coli* SPase I produced in *B. subtilis* was not active.

Discussion

Recently, we demonstrated that among a collection of precursors of hybrid secretory proteins, which contained signal peptides randomly selected from the *B. subtilis* chromosome, several were processed relatively slowly in *B. subtilis* and *E. coli* (Smith *et al.*, 1989; H. Smith and others, unpublished; van Dijl *et al.*, 1991*b*). We have demonstrated before that the rates of processing of two of these precursors [pre(A2d)-β-lactamase and pre(A13i)-β-lactamase] could be increased by overproduction of SPase I in *E. coli* (van Dijl *et al.*, 1991*a*). This indicated that in *E. coli*, under standard conditions, the availability of SPase I was rate-limiting for the processing of these precursors. By analogy, we reasoned that the inefficient processing of two precursors [pre(A13)-α-amylase and pre(A2d)-β-lactamase] in *B. subtilis* might also be due to limited availability of SPase.

To test this, we expressed the *E. coli lep* gene in *B. subtilis* by fusing it to appropriate transcription/translation signals. As a consequence of this construction, the *E. coli* SPase I contained seven additional amino acids at its N-terminus. Apparently, this extension did not interfere with the activity of the enzyme in *E. coli*. In contrast, our results suggest that activity of the *E. coli* SPase I was absent in *B. subtilis in vivo*. Even *in vitro*, after solubilization in Triton X-100, the activity could not be recovered. This lack of processing activity did not appear to result from inhibiting components in *B. subtilis*. This indicates that the *E. coli* SPase I itself was inactive in *B. subtilis*. Two explanations may be considered to underlie this phenomenon. The first is that the inactivity of the *E.*

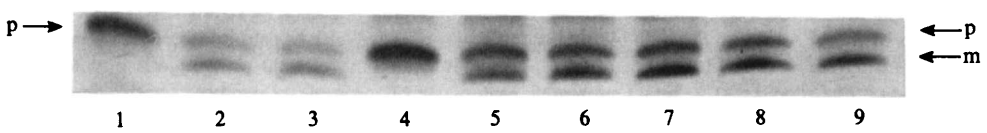


Fig. 8. Lack of interference of cell-free *B. subtilis* extracts with the *in vitro* processing-activity of *E. coli* SPase I. *In vitro* synthesized bacteriophage M13 procoat was incubated with cell-free extracts of *E. coli* C600 (0·4 mg protein ml^{-1}), *B. subtilis* DB114 (4 mg protein ml^{-1}), or mixtures of these. Lanes: 1, no addition; 2, *E. coli* C600; 3, *E. coli* C600(pTD101); 4, *B. subtilis* DB104; 5, *B. subtilis* DB104 plus *E. coli* C600; 6, *B. subtilis* DB104 plus *E. coli* C600(pTD101); 7, *B. subtilis* DB104 plus purified SPase I; 8, *E. coli* C600 plus purified SPase I; 9, purified SPaseI only. p, Precursor; m, mature.

coli SPase I produced in *B. subtilis* is due to incorrect folding of the enzyme. We can only speculate about possible reasons for inappropriate folding. One is that particular cellular components, such as chaperones (see Lecker *et al.*, 1989; Saier *et al.*, 1989), are necessary to guide the correct folding of the *E. coli* SPase I. It is conceivable that the corresponding components of *B. subtilis* are not able to effect the correct folding of the heterologous protein. An alternative reason is that in *B. subtilis* the seven additional N-terminal amino acids of the *E. coli* SPase I fusion protein interfered with the folding of the protein into the conformation required for activity. However, this explanation is not easily reconciled with the observation that in *E. coli* the fusion protein was active in the complementation assay.

As a second possible explanation for the lack of *E. coli* SPase I activity in *B. subtilis*, incorrect insertion of the protein into the membrane can be considered. In *E. coli* the correct assembly of SPase I results in the exposure of the C-terminal part (two-thirds of the total protein) on the outer surface of the cytoplasmic membrane. As a consequence, after spheroplasting the enzyme is sensitive to trypsin (Moore & Miura, 1987; Wolfe *et al.*, 1983*a*), Mechanistically, the membrane insertion of SPase I appears, at least in part, to share features with the export of proteins. It requires the (internal) signal peptide of the protein (Dalbey *et al.*, 1987; Zhu & Dalbey, 1989) and the activities of the SecA and SecY proteins (Wolfe *et al.*, 1985).

The present results indicated that the *E. coli* SPase I was associated with the membrane when expressed in *B. subtilis*. However, in protoplasts, the protein was protected against degradation by trypsin, indicating that the enzyme was not inserted correctly into the *B. subtilis* cytoplasmic membrane. Although a positive control showing that trypsin under these conditions could degrade other *B. subtilis* membrane proteins was not carried out, these data suggest that the SPase I was either inserted across the membrane without exposing a proper cleavage site for trypsin at the external surface, or that it was associated with the cytoplasmic side of the membrane. Obviously, this would prevent the correct interaction between precursors and SPase I. Alternatively, the SPase I may have formed aggregates in the cytoplasm, which fractionated with the membranes. If defective membrane insertion has occurred in this system, this might be of more general importance. It could mean that the apparatus for membrane insertion is host-specific and is crucial for the final structure of proteins in the membrane. The causes of the presumed defective membrane insertion are not clear. One possibility is that the heterologous protein does not interact efficiently with essential components of the *B. subtilis* export machinery (e.g. chaperones, SecA or SecY), preventing the correct translocation of the protein across the membrane.

The incorrect membrane assembly of the *E. coli* SPase I in *B. subtilis* is reminiscent to the defective export of the *E. coli* outer-membrane protein A (OmpA) in *B. subtilis* (Kallio *et al.*, 1986). OmpA, when fused to the signal peptide of *B. amyloliquefaciens* α-amylase, was not translocated across the cytoplasmic membrane of *B. subtilis*. Kallio *et al.* (1986) suggested that this may be due to the absence in *B. subtilis* of a specific factor required for the translocation of OmpA across the membrane.

One interesting outcome of the present work is that the *B. subtilis* and *E. coli* SPase I proteins appear to differ in two aspects. First, antibodies raised against *E. coli* SPase I did not cross-react with *B. subtilis* membrane proteins. Second, the *B. subtilis* SPase I did not show *in vitro* processing activity under conditions appropriate for *E. coli* SPase I. Two other lines of evidence also suggest that the SPases of Gram-positive bacteria may be rather distinct from those of Gram-negative bacteria. First, the *lep* genes of *E. coli* and *Salmonella typhimurium* showed no similarity with the *B. licheniformis lep* gene (J. M. van Dijl and others, unpublished). Second, *B. stearothermophilus* α-amylase is processed at different sites in *B. subtilis* and in *E. coli* (Suominen *et al.*, 1987).

For a better understanding of the differences between SPases from Gram-positive and Gram-negative bacteria, the cloning and analysis of a *Bacillus* SPase I is required. This will also enable us to address the question whether the availability of this enzyme can be limiting for the secretion of proteins by this organism.

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